

Chem 460 Laboratory—Fall 2009
Experiment 5: Purification of Hen Egg White Lysozyme
~Week 2~

BCA Protein Assay

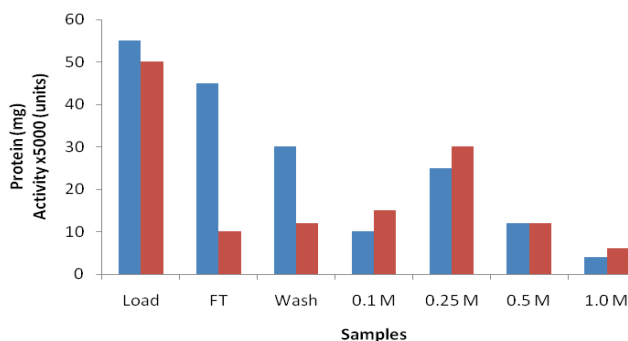
To determine the amount of protein in your various samples, we will use the spectrophotometric [Pierce \(http://www.piercenet.com/\)](http://www.piercenet.com/) BCA Protein Assay kit which consists of 2.0 mg/mL standard solutions of BSA (Bovine Serum Albumin, which should not be confused with BCA, which is bicinchoninic acid), Reagent A and Reagent B. An Instruction Manual (<http://www.piercenet.com/files/1296as8.pdf>) is available from their website and you need to access it for information on how the assay works.

We will adapt the standard protocol to be used in microcentrifuge tubes (final volume 900 μ L). We measure the protein concentration in our **samples** by constructing a **standard** curve. Start by making five standard solutions with concentrations starting at 0 mg/ml BSA and going up to 1 mg/mL BSA, each in a final volume of 100 μ L. To do this you need to dilute the 2 mg/mL BSA stock. I recommend doing your standards in duplicate since the standard curve should be very accurate.

Next, you need to set up your samples. You should have at least 6 samples: Load, FT, Wash, and Fractions 1-3 (or more). We don't know *a priori* how much of each of these we need so that our samples fit onto our standard curve, but 10 μ L of sample is a reasonable amount to try. The final volume for each sample should be 100 μ L (dilute with water) and they should be done in duplicate too.

Next, you need to make the “assay cocktail” which involves mixing 50 parts Reagent A with 1 part Reagent B. Since you will add 800 μ L of the cocktail to each **standard** and **sample**, you need to determine the total volume of cocktail you need to make. Keep in mind that the assay is colorimetric, which means that you need to add the cocktail to the standards and samples **at about the same time**, otherwise their colors won't be comparable. Allow the color to develop ~15 minutes at 37° C (watch it carefully and ask your lab instructor how long to wait), put your samples on ice, and then measure A_{562} for each standard and sample.

Once you have determined the concentration of protein in your samples, enter this information into the purification table you began during Week 1. In addition, determine the total amount of protein in the entire (i.e. 10 mL) egg-white extract. Keep in mind any dilutions you made in this assay. Using this data, create a plot of protein (mg) and activity (units) vs. sample (see below). Adjust your axes so that these values are comparable. This plot provides a nice visual depiction of the purification.



Trypsin Digestion

In Week 3 we will analyze by liquid chromatography mass spectrometry (LCMS) the lysozyme you purified in Week 1. As a lab, we will be carrying out an MS on the full intact protein and also an MS/MS on peptides of it. For this latter analysis, you will choose a fraction with high lysozyme specific activity and digest it with the enzyme trypsin. Lysozyme is a hearty extracellular enzyme with disulfide bonds; therefore the protocol below is required prior to MS/MS analysis. Since this procedure requires almost 2 hours to complete, you should start it as soon as possible.

In-Solution Digestion (modified from <http://www.osa.sunysb.edu/Proteomics/ProteinDigestPrep.pdf>)

Solutions

1. 0.4 M Tris stock at pH 7.8 (10mL)
2. 6 M urea in 100 mM Tris buffer: Place 2.0 g of urea in a 15-mL centrifuge tube. Add 1.25 mL of the Tris stock. Adjust the total volume to 5 mL with water.
3. 200 mM DTT in 100 mM Tris: Dissolve 30 mg of DTT in 750 μ L of water. Add 250 μ L of Tris stock.
4. 200 mM iodoacetamide in 100 mM Tris: Dissolve 36 mg of iodoacetamide in 750 μ L of water. Add 250 μ L of the Tris stock.
5. 200 ng/ μ L Trypsin solution: Add 25 μ L of ice-cold Tris stock and 75 μ L of ice-cold water to 20 μ g of trypsin. Dissolve the trypsin by drawing the solution into and out of the pipette. Keep the solution on ice until use.

Digestion (35 μ L total volume)

1. Add 10- μ L of 6 M urea (in 100 mM tris buffer) to 100 μ g of total protein, in a 1.5-mL plastic microcentrifuge tube.
2. Add 0.5 μ L of the reducing reagent (DTT) and mix the sample by gentle vortex. Let sit at room temperature for ~30 minutes.
3. Add 2 μ L of the alkylating reagent (iodoacetamide) and mix the sample by gentle vortex. Let sit at room temperature for ~30 minutes.
4. Add 2 μ L of the reducing agent to consume any unreacted iodoacetamide. Mix the sample by gentle vortex and allow the reaction to stand at room temperature for ~15 minutes.
5. Reduce the urea concentration by diluting the reaction mixture with 85 μ L of water. Mix the solution by gentle vortex. This dilution reduces the urea concentration to ~0.6 M, a concentration at which the trypsin retains its activity.
6. Add 4 μ g of trypsin. Mix the sample by gentle vortex and carry out the digestion overnight at 37 °C. Your instructor will then put your digests at -20°C until next week.